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(54) Title: A METHOD FOR THE TARGETING OF PROTEINS PRODUCED BY *YERSINIA* INTO THE CYTOSOL OF EUKARYOTIC CELLS

(57) Abstract

A method of delivering a polypeptide to eukaryotic cells based on the YopE system of *Yersinia* involves one step translocation of the polypeptide in the response to targeting signals provided by the first 100 amino acid residues of the YopE protein of the first 100 amino acid residues of the YopE protein of *Yersinia*. This method requires functional SycE chaperone activity. The translocated polypeptide is delivered to the cytosol of the eukaryotic cells. In general, this method comprises: (1) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (a) the composite YopE targeting signal; and (b) a polypeptide to be translocated, the nucleic acid segment capable of expression in *Yersinia* cells; (2) expressing the nucleic acid segment in *Yersinia* cells; and (3) contacting *Yersinia* cells expressing the nucleic acid segment which eukaryotic cells in the presence of functional SycE chaperone activity so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells. The method is not limited to *Yersinia* cells, but can be used with other Gram–negative bacteria as well. The present invention also includes screening methods and methods of blocking the introduction of a bacterial protein translocated by a translocation system in a Gram–negative bacterium employing a targeting signal into a eukaryotic cell.

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# A METHOD FOR THE TARGETING OF PROTEINS PRODUCED BY *YERSINIA* INTO THE CYTOSOL OF EUKARYOTIC CELLS

## BACKGROUND OF THE INVENTION

This invention is directed to a method for targeting proteins produced by *Yersinia* to the cytosol of eukaryotic cells and targeting signals that can be used in these methods.

For many purposes, it is desirable to target proteins, particularly recombinant proteins, produced by bacteria to the cytosol of eukaryotic cells. These proteins can be targeted for diagnostic or therapeutic purposes. Among the proteins that can be targeted are antibodies, toxins, receptor proteins, and polypeptide hormones.

However, there is a need for an improved and more efficient and widely applicable method for targeting proteins to the cytosol of eukaryotic cells. Preferably, such a method would not depend on the specific structural features of the proteins to be targeted and could be used with virtually any fusion protein and with a broad range of eukaryotic cells. Preferably, such a method preserves the three-dimensional structure and activity of the translocated proteins and is performed under conditions that do not expose such proteins to denaturing or destabilizing conditions. Preferably, such a method is also suitable for a broad range of applications of the translocated proteins, such as screening.

## SUMMARY

Here, we have developed a fractionation assay for *Y. enterocolitica* infected HeLa cells to measure Yop secretion and targeting. Yops were located in either the eukaryotic cytosol, the extra-cellular medium or remained associated with the bacteria. We could not detect an extra-cellular intermediate for those Yops that were localized to the HeLa cytosol. Targeting depended on the binding of SycE chaperone to YopE residues 15-100 in the *Yersinia* cytoplasm. During infection of HeLa cells, the signal for YopE

export in low calcium-induced *Yersiniae* (codons 1-15) did not lead to secretion into the extracellular medium. We propose a novel targeting mechanism that directs Yop proteins from the bacterial cytoplasm directly into the cytosol of eukaryotic cells. This fractionation assay allows us to provide methods for the targeting of proteins produced by Yersinia directly into eukaryotic cells.

In general, employing *Yersinia*, a method according to the present invention comprises:

(1) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (a) a composite YopE targeting signal; and (b) a polypeptide to be translocated, the nucleic acid segment being expressed in *Yersinia* cells;

(2) expressing the nucleic acid segment in *Yersinia* cells;

(3) contacting the *Yersinia* cells expressing the nucleic acid segment with eukaryotic cells in the presence of functional SycE chaperone activity so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells.

Typically, the *Yersinia* species is *Y. enterocolitica* or *Y. pseudotuberculosis*. Preferably, the *Yersinia* species employed is *Y. enterocolitica*.

The eukaryotic cells can be animal cells, such as mammalian cells, including human cells. The eukaryotic cells can be of a cell type selected from the group consisting of fibroblasts, epithelial cells, and leukocytes.

Alternatively, the eukaryotic cells can be plant cells.

The polypeptide can be selected from the group consisting of an antibody, an enzyme, a receptor protein, a hormone, and a toxin. The polypeptide can further comprise a reporter segment.

In one preferred alternative, the composite targeting signal comprises the amino acid sequence M-K-I-S-S-F-I-S-T-S-L-P-L-P-A-S-V-S-G-S-S-S-V-G-E-M-S-G-R-S-V-S-Q-Q-K-S-D-Q-Y-A-N-N-L-A-G-R-T-E-S-P-Q-G-S-S-L-A-S-R-I-I-E-R-L-S-S-M-A-

H-S-V-I-G-F-I-Q-R-M-F-S-E-G-S-H-K-P-V-V-T-P-A-L-T-P-A-Q-M-P-S-P-T (SEQ ID NO: 1). More generally, the first fifteen amino acids of the composite targeting signal can be M-K-I-S-S-F-I-S-T-S-L-P-L-P-A (SEQ ID NO: 1). Also more generally, residues 16-100 of the composite targeting signal can be selected from the group consisting of:

(1) S-V-S-G-S-S-S-V-G-E-M-S-G-R-S-V-S-Q-Q-K-S-D-Q-Y-A-N-N-L-A-G-R-T-E-S-P-Q-G-S-S-L-A-S-R-I-I-E-R-L-S-S-M-A-H-S-V-I-G-F-I-Q-R-M-F-S-E-G-S-H-K-P-V-V-T-P-A-L-T-P-A-Q-M-P-S-P-T, which are residues 16-100 of SEQ ID NO: 1; and

(2) sequences that include are modified from residues 16-100 of SEQ ID NO: 1 by one or more of the following conservative amino acid substitutions:

(a) isoleucine for either valine or leucine;

(b) valine for either isoleucine or leucine;

(c) leucine for either valine or isoleucine;

(d) aspartic acid for glutamic acid;

(e) glutamic acid for aspartic acid:

(f) glutamine for asparagine;

(g) asparagine for glutamine;

(h) serine for threonine; and

(i) threonine for serine.

In another preferred alternative, the first fifteen amino acids of the composite targeting signal are encoded by a mRNA sequence of AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCA (SEQ ID NO: 2).

Alternatively, the first fifteen amino acids of the composite targeting signal are encoded by a mRNA sequence that is selected from the group consisting of:

(1) the mRNA sequence of AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCA (SEQ ID NO: 2); and

(2) a mRNA sequence that is modified from the sequence AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCA by a

frameshift mutation, with the proviso that the hydrophobicity of the segment of the protein encoded by the mRNA including therein any frameshift mutation occurring within the sequence AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCA is substantially equivalent to the hydrophobicity of the segment of the protein encoded by SEQ ID NO: 2.

In another alternative, the first fifteen amino acids of the targeting signal can be encoded by a mRNA including the sequence AAAAUAU that is part of the first loop of SEQ ID NO: 2.

Although the method is being described herein with particular reference to *Yersinia*, the method can also be used with other Gram-negative bacterial cells possessing a homologous or substantially homologous translocation mechanism, one designated as Type III. These Gram-negative bacterial cells include *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Pseudomonas spp.*, and *Xanthomonas spp*. Thus, another aspect of the present invention is a method for delivering a peptide to the cytosol of a eukaryotic cell comprising the steps of:

(1) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (a) a targeting signal effective for targeting in a Gram-negative bacterium possessing a Type III translocation mechanism homologous or substantially homologous to the translocation mechanism of *Yersinia*; and (b) a polypeptide to be translocated, the nucleic acid segment being expressed in the cells of the Gram-negative bacterium;

(2) expressing the nucleic acid segment in the Gram-negative bacterial cells;

(3) contacting the Gram-negative bacterial cells expressing the nucleic acid segment with eukaryotic cells in the presence of functional chaperone activity homologous or substantially homologous to SycE chaperone activity of *Yersinia* so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells.

In this more generalized method, the targeting signal can include a second portion that is specific for a protein that has substantial homology with the SycE protein of *Yersinia* at its carboxy-terminal end and has chaperone function.

Another aspect of the present invention is a method for blocking the introduction of a bacterial protein translocated by a translocation system in a Gram-negative bacterium employing a targeting signal into a eukaryotic cell comprising inhibiting the transcription or translation of mRNA for a protein of the translocation system having protein targeting activity by administering at least one antisense oligonucleotide to inhibit the transcription or translation of the protein having protein targeting activity. In one preferred alternative, the Gram-negative bacterium is a *Yersinia* species and the protein of the translocation system is the *Yersinia* YopE protein.

Yet another aspect of the present invention is a method for screening for the activity of a translocated polypeptide in a eukaryotic cell. The method comprises:

(1) providing a translocated polypeptide in a eukaryotic cell by:

(a) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (i) a composite YopE targeting signal; and (ii) a polypeptide to be translocated, the nucleic acid segment being expressed in *Yersinia* cells;

(b) expressing the nucleic acid segment in *Yersinia* cells;

(c) contacting the *Yersinia* cells expressing the nucleic acid segment with eukaryotic cells in the presence of functional SycE chaperone activity so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells; and

(2) screening for the activity of the translocated polypeptide by detecting the result of an activity of the polypeptide in the eukaryotic cell.

The detection of the result of the activity of the polypeptide in the eukaryotic cell can be performed by a method selected from the group consisting of detection of an enzymatic activity, detection of an antibody activity, and detection of a specific binding activity.

The eukaryotic cells into which the fusion protein including the polypeptide is to be translated can be animal or plant cells. If animal cells, they can be mammalian or non-mammalian cells, including amphibian, fish, reptilian, or avian cells. If they are mammalian cells, they can be human or non-human cells. If they are non-human cells, they can be primate or non-primate cells, including feline, canine, bovine, ovine, equine, murine, or other

non-primate cells. The method can be used with a large range of cell types, including fibroblasts, epithelial cells, and leukocytes.

Another aspect of the present invention is a method for screening for the activity of a translocated polypeptide in a eukaryotic cell. This method comprises:

(1) providing a translocated polypeptide in a eukaryotic cell by the method described above; and

(2) screening for the activity of the translocated polypeptide by detecting the result of an activity of the polypeptide in the eukaryotic cell.

Because of the pathogenicity of many Gram-negative bacteria, the translocation process described in the present application represents a novel site for antibiotic action. Accordingly, a process for blocking the introduction of bacterial proteins into eukaryotic cells is within the scope of the present invention. This process can involve inhibiting the transcription or translation of the mRNA for the YopE protein or an analogous protein with antisense technology.

## BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims, and accompanying drawings where:

Figure 1 shows the localization of Yop proteins during HeLa cell infections of *Y. enterocolitica*; media (Med.) were decanted from HeLa cells and centrifuged to sediment non-adherent bacteria (P, pellet) and separate them from secreted Yops in the supernatant (S); HeLa cells and attached *Yersiniae* were extracted with digitonin (Dig.) to specifically solubilize the HeLa plasma membrane and release Yop proteins targeted into the eukaryotic cytosol; unlysed bacteria, eukaryotic membranes and organelles were sedimented by centrifugation (P) and separated from the HeLa cytosol in the supernatant (S); as a control for solubilization of all membranes, infected HeLa cells were extracted with SDS followed by centrifugation; proteins were precipitated with chloroform / methanol, separated on SDS-

PAGE, electroblotted and immuno-stained with specific antisera; infections were with either, A. wild-type *Y. enterocolitica* W22703 or B. yopNI mutant strain VTLI; *Yersinia* Yop proteins, SycE, and CAT as well as HeLa cell cytosolic FPT and nuclear TFIID were analyzed; for wild-type *Yersinia*, 36% (±6) of YopE, 77% (±9) of YopH, 77% (±21) of YopM, and 7% (±10) of YopN were soluble after digitonin extraction; standard deviations () were calculated from at least four independent measurements;

Figure 2 shows the targeting signal of YopE: A. translational fusions of YopE to neomycin phosphotransferase (NPT) were analyzed for their targeting into the eukaryotic cytosol; B. wild-type Y enterocolitica strain W22703 (*sycE*$^+$, *yopN*$^+$); C. *sycE1* mutant LC2 (*sycE*$^-$, *yopN*$^+$); and D. *yopN1* mutant VTLI (*sycE*$^+$, *yopN*$^-$) expressing YopE-NPT translational fusions infected HeLa tissue cultures for 3 hours prior to digitonin fractionation as described in the legend to Fig. 1; targeting was measured as the percent amount of digitonin solubilized YopE-NPT fusion: B. YopE-NPT 34%, $YopE_{1-100}$-NPT 81%, $YopE_{1-50}$-NPT 0%, $YopE_{1-15}$-NPT 0% and NPT 0%; C. No targeting (0%) was observed for all YopE-NPT fusions in *sycE1* strain LC2; D. the *yopN1* mutant strain VTLI secreted YopE-NPT fusions into the surrounding medium; as a control, $YopE_{1-15,+1}$-NPT (pDA72) harboring a mutant secretion signal as well as NPT alone (pDA35) remained in the cytoplasm;

Figure 3 shows immunofluorescent detection of YopE-NPT fusions by confocal laser microscopy; HeLa cells were infected with *Y enterocolitica* strains expressing YopE-NPT fusion proteins and targeting was detected by indirect immunofluorescence using anti-NPT and Oregon green-conjugated secondary antibody; the HeLa plasma membrane was stained with Texas red-conjugated wheat germ agglutinin. Panels show infections of *Y. enterocolitica* W22703 harboring A. pDA36 (YopE-NPT), B. pDA44 ($YopE_{1-100}$-NPT), C pDA45 ($YopE_{1-50}$-NPT), D. pDA46 ($YopE_{1-15}$-NPT), E. pDA72($YopE_{1-15+1}$-NPT), F. *sycE1* strain LC2 expressing YopE-NPT (pDA36), G. W22703 pDA35 (NPT), and H. W22703 no plasmid control;

Figure 4 shows the substrate requirements for YopE secretion and targeting by the type III machinery; YopE-NPT fusion proteins were expressed in either wild-type *Y. enterocolitica* W22703 or *sycE1* mutant strain LC2; targeting of YopE-NPT fusions during the infection of HeLa cells was performed as described in the legend to Fig. 1; digitonin extracted cells were centrifuged to separate the supernatant (S), representing the HeLa

cytosol, from the sedimented pellet (P) containing intact bacteria; secretion was measured by low calcium and temperature induced type III export of YopE-NPT proteins into the culture medium (-$Ca^{2+}$); cultures were centrifuged and sedimented *Yersiniae* (P) were separated from the supernatant (S) containing secreted Yops; samples were precipitated with either chloroform / methanol (targeting) or TCA / acetone (secretion) and analyzed by immunoblotting; fusions were constructed by replacing the first fifteen codons of *yopE*, specifying its secretion signal, with those of chloramphenicol acetyl-transferase (*cat*)(pVL33), β-galactosidase (lacZ)(pVL35), and a +1 frame shift mutation (pDA141) of the wild-type *yopE* secretion signal (*yopE+1*)(pDA139); and

Figure 5 shows immuno-electron microscopic detection of YopE-NPT targeting; HeLa cells were infected with *Y. enterocolitica* W22703 (pDA36) as described in the legend to Fig. 1 and processed for transmission electron microscopy; thin sections were incubated with antiNPT and protein A-colloidal gold conjugate (9 nm) followed by staining with uranyl acetate and lead; see Table 1 for quantification of gold particles.

## DEFINITIONS

As used herein, the terms defined below have the following meanings unless otherwise indicated:

"Nucleic Acid Sequence": the term "nucleic acid sequence" includes both DNA and RNA unless otherwise specified, and, unless otherwise specified, includes both double-stranded and single-stranded nucleic acids. Also included are hybrids such as DNA-RNA hybrids. In particular, a reference to DNA includes RNA that has either the equivalent base sequence except for the substitution of uracil and RNA for thymine in DNA, or has a complementary base sequence except for the substitution of uracil for thymine, complementarity being determined according to the Watson-Crick base pairing rules. Reference to nucleic acid sequences can also include modified bases as long as the modifications do not significantly interfere either with binding of a ligand such as a protein by the nucleic acid or with Watson-Crick base pairing.

"Antibody": as used herein the term "antibody" includes both intact antibody

molecules of the appropriate specificity, and antibody fragments (including Fab, F(ab'), Fv, and $F(ab')_2$), as well as chemically modified intact antibody molecules and antibody fragments, including hybrid antibodies assembled by *in vitro* reassociation of subunits. Also included are single-chain antibody molecules generally denoted by the term sFv and humanized antibodies in which some or all of the originally non-human constant regions are replaced with constant regions originally derived from human antibody sequences. Both polyclonal and monoclonal antibodies are included unless otherwise specified. Additionally included are modified antibodies or antibodies conjugated to labels or other molecules that do not block or alter the binding capacity of the antibody.

## DESCRIPTION

We have developed a method of delivering a polypeptide to eukaryotic cells based on the YopE system of *Yersinia*. The method involves one step translocation of the polypeptide in response to targeting signals provided by the first 100 amino acid residues of the YopE protein of the first 100 amino acid residues of the YopE protein of *Yersinia*. This method requires functional SycE chaperone activity. The translocated polypeptide is delivered to the cytosol of the eukaryotic cells.

The improved way of targeting proteins to the cytosol of eukaryotic cells according to the present invention makes use of targeting mechanisms employed by pathogenic bacteria. One such class of pathogenic bacteria is the genus *Yersinia*. Upon entering their human host, most bacteria are phagocytosed and killed by the cellular immune system. Three pathogenic *Yersinia* species, *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*, have devised a mechanism that, upon direct contact, kills host cells and allows these microbes to multiply within lymphoid tissues and to establish disease (Straley et al., 1993a; Cornelis & Wolf-Watz, 1997). Contact with eukaryotic cells provides a signal for *Yersinia* to target cytotoxic proteins, Yops (*Yersinia* out proteins), into the host cells by a type III mechanism (Rosqvist et al, 1994; Petterson et al, 1996). Once within the eukaryotic cytosol, Yop proteins interfere with signal transduction and cytoskeletal rearrangement

events, thereby allowing *Yersinia* to evade the infected host's defense (Bliska et al, 1991; Galyov et al., 1993; Straley et al., 1993b).

In the absence of eukaryotic cells, type III secretion can also be induced by low calcium concentration and temperature shift to 37°C, which results in the secretion of fourteen different Yop proteins into the surrounding medium (Michiels et al, 1990). (Herein, secretion is defined as type III export of Yops into the extra-cellular medium, whereas targeting refers to the localization of Yops into the eukaryotic cytosol.) The signal sufficient for the secretion of reporter fusions in low calcium-induced cultures has been mapped to the first fifteen codons of Yops (Michiels and Cornelis, 1991; Sory et al., 1995; Schesser et al., 1996). This signal is likely encoded within *yop* mRNA, because frameshift mutations that completely alter its protein sequence promote secretion of the fused reporter proteins (Anderson and Schneewind, 1997). A second independent type III export pathway has been revealed for YopE (Cheng et al., 1997). Mutant YopE with a defective secretion signal can be exported in a manner absolutely dependent on the presence of SycE chaperone. SycE is a small homodimeric protein that binds to residues 15- 1 00 of YopE in the *Yersinia* cytoplasm (Wattiau and Cornelis, 1993; Woestyn *et al.*, 1996) and this interaction is also sufficient to initiate YopE into the secretary pathway (Cheng et al., 1997).

Targeting of Yops into the cytosol of eukaryotic cells has first been observed with immunofluorescence microscopy: YopE, YopH and YpkA (YopO) were found in the cytosol of HeLa cells that had been infected with Y pseudotuberculosis (Rosqvist et al., 1994; Persson et al., 1995; Hakansson et al., 1996b). In another experiment, *Y. enterocolitica* were manipulated to express Yop fusions to *Bordetella* pertussis adenylate cyclase (Cya) which resulted in an increase of cAMP in the eukaryotic cytosol (Sory and Cornelis, 1994; Sory et al., 1995). The increase of cAMP is thought to be a measure of Yop targeting, because *Bordetella* adenylate cyclase absolutely requires calmodulin in the eukaryotic cytosol for enzymatic activity. Fusions of YopE, YopH, and YopM to adenylate cyclase increased cytosolic cAMP levels, whereas YopN fusions did not (Boland *et al.*, 1996). Shorter N-terminal Yop segments (YopE 1-50, YopH 1-71, or YopM 1-130) also increased cAMP levels, but fusions to the first fifteen codons of Yops did not have this effect (Sory et al., 1995). Equal amounts of hybrid Cya proteins were found in the extracellular

medium and in eukaryotic cell lysates, suggesting that the extra-cellular Yops may represent an intermediate step of Yop targeting (Boland et al., 1996). A specific role of Syc proteins in Yop targeting has hitherto not been demonstrated (Frithz-Lindsten et al., 1995).

On the basis of these observations, Yop targeting was proposed to occur by a two step mechanism (Sory et al., 1995). Yops may first be exported across the bacterial envelope via their secretion signal (codons 1-15). The translocation domain of YopE, YopH and YopM (residues 15-50, 15-71 15-130 respectively) may then target these polypeptides from the medium or the bacterial surface into the eukaryotic cytosol (Sory et al., 1995; Boland et al., 1996).

In general, employing *Yersinia*, this method comprises:

(1) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (a) the composite YopE targeting signal; and (b) a polypeptide to be translocated, the nucleic acid segment capable of expression in *Yersinia* cells;

(2) expressing the nucleic acid segment in *Yersinia* cells; and

(3) contacting the *Yersinia* cells expressing the nucleic acid segment with eukaryotic cells in the presence of functional SycE chaperone activity so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells.

Typically, the *Yersinia* species is *Y. enterocolitica* or *Y. pseudotuberculosis*. Preferably, the *Yersinia* species employed is *Y. enterocolitica*.

Although the method is being described herein with particular reference to *Yersinia*, the method can also be used with other Gram-negative bacterial cells possessing a homologous or substantially homologous translocation mechanism, one designated as Type III. These Gram-negative bacterial cells include *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Pseudomonas spp.*, and *Xanthomonas spp.* Therefore, within the scope of the present invention are processes employing these Gram-negative bacteria. For convenience, the method is described herein employing *Yersinia*, but this is not intended to exclude the use of other appropriate Gram-negative bacteria.

In general, this method comprises:

(1) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (a) a targeting signal effective for targeting in a Gram-negative bacterium possessing a Type III translocation mechanism homologous or substantially homologous to the translocation mechanism of *Yersinia*; and (b) a polypeptide to be translocated, the nucleic acid segment being expressed in the cells of the Gram-negative bacterium;

(2) expressing the nucleic acid segment in the Gram-negative bacterial cells;

(3) contacting the Gram-negative bacterial cells expressing the nucleic acid segment with eukaryotic cells in the presence of functional chaperone activity homologous or substantially homologous to SycE chaperone activity of *Yersinia* so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells.

A significant degree of homology between the *Yersinia* SycE protein and other bacterial chaperones exists at the carboxy-terminal end (Wattiau et al., 1996). Accordingly, within the scope of the present invention are methods employing targeting signals whose second portions are specific for proteins that have substantial homology with SycE at the carboxy-terminal end and have chaperone function. These methods are for use in Gram-negative bacteria such as *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Pseudomonas spp.*, and *Xanthomonas spp.* according to the general method for such bacteria described above.

The eukaryotic cells into which the fusion protein including the polypeptide is to be translated can be animal or plant cells. If animal cells, they can be mammalian or non-mammalian cells, including amphibian, fish, reptilian, or avian cells. If they are mammalian cells, they can be human or non-human cells. If they are non-human cells, they can be primate or non-primate cells, including feline, canine, bovine, ovine, equine, murine, or other non-primate cells. The method can be used with a large range of cell types, including fibroblasts, epithelial cells, and leukocytes.

Methods for preparing the nucleic acid segment are well known in the art and need not be described in detail further here. Typically, the nucleic acid segment encoding the polypeptide to be translocated is DNA.

Typically, the nucleic acid segment encoding the polypeptide to be translocated is cloned into the *Yersinia*. However, other methods involving transient expression are also possible and the method of the present invention is not limited to cloned nucleic acid segments.

Cloning the nucleic acid segment encoding the fusion protein into the *Yersinia* is performed by standard methods. In general, such cloning involves: (1) isolation of a nucleic acid segment encoding the polypeptide to be translocated; (2) joining the nucleic acid segment to the composite YopE targeting signal; (3) cloning by insertion into a vector compatible with the bacterium in which expression is to take place; and (4) incorporation of the vector including the new chimeric nucleic acid segment into the bacterium.

Typically, the nucleic acid segment encoding the protein to be sorted is DNA; however, the use of RNA in certain cloning steps is within the scope of the present invention.

When dealing with genes from eukaryotic organisms, it is preferred to use cDNA, because the natural gene typically contains intervening sequences or introns that are not translated. Alternatively, if the amino acid sequence is known, a synthetic gene encoding the protein to be sorted can be constructed by standard solid-phase oligodeoxyribonucleotide synthesis methods, such as the phosphotriester or phosphite triester methods. The sequence of the synthetic gene is determined by the genetic code, by which each naturally occurring amino acid is specified by one or more codons. Additionally, if a portion of the protein sequence is known, but the gene or messenger RNA has not been isolated, the amino acid sequence can be used to construct a degenerate set of probes according to the known degeneracy of the genetic code. General aspects of cloning are described, for example, in J. Sambrook et al., "Molecular Cloning: A Laboratory Manual" (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989); in B. Perbal, "A Practical Guide to Molecular Cloning" (2d ed., John Wiley & Sons, New York 1988), in S.L. Berger & A.R. Kimmel, "Guide to Molecular Cloning Techniques" (Methods in Enzymology, vol. 152, Academic Press, Inc., San Diego, 1987), and in D.V. Goeddel, ed., "Gene Expression Technology" (Methods in Enzymology, vol. 185, Academic Press, Inc., San Diego, 1991).

Once isolated, DNA encoding the protein to be sorted is then joined to the composite YopE targeting signal. This is typically accomplished through ligation, such as using *Escherichia coli* or bacteriophage T4 ligase. Conditions for the use of these enzymes are well known and are described, for example, in the above general references.

The ligation is done in such a way so that the protein to be sorted and the sorting signal are joined in a single contiguous reading frame so that a single protein is produced. This may, in some cases, involve addition or deletion of bases of the cloned DNA segment to maintain a single reading frame. This can be done by using standard techniques.

Cloning is typically performed by inserting the cloned DNA into a vector containing control elements to allow expression of the cloned DNA. The vector is then incorporated into the bacterium in which expression is to occur, using standard techniques of transformation or other techniques for introducing nucleic acids into bacteria. The control elements can be such elements as promoters or operators. These elements and methods for their use are well known in the art and need not be recited further here.

The polypeptide to be translocated can be any polypeptide capable of expression in a fusion protein. For example, but not by way of limitation, the polypeptide can be an antibody, an enzyme, a receptor protein, a hormone, a toxin, or any other polypeptide having a biological or physiological activity. The polypeptide can itself be the result of a previous fusion and thus can carry a "tag" or reporter segment.

Accordingly, another aspect of the present invention is a method for screening for the activity of a translocated polypeptide in a eukaryotic cell. This method comprises:

(1) providing a translocated polypeptide in a eukaryotic cell by the method described above; and

(2) screening for the activity of the translocated polypeptide by detecting the result of an activity of the polypeptide in the eukaryotic cell. The detection of the result of an activity of the polypeptide can be the detection of an enzymatic activity, an antibody activity, or a specific binding activity, such as those mediated by hormones, toxins, receptor

proteins, or other proteins having biological or physiological activity . In this context the term "activity" refers to all reactions that depend on the three-dimensional structure of the protein and are specific to proteins with that three-dimensional structure. The detection of the activity can be done by methods well known in the art, such as the detection of a product of a reaction catalyzed by the protein if the protein has enzymatic activity, the detection of the detection of a labeled specific binding partner, such as an antibody or a small molecule of the appropriate specificity, specifically binding to the polypeptide, or the detection of an effect caused by the introduction of the polypeptide, such as growth or differentiation of the cell into which the polypeptide has been introduced.

The composite YopE targeting signal comprises two regions, both of which are required to be present. The first region is the first 15 amino acids of the signal, which is required for the targeting even though it is not required for YopE-directed secretion. (Cheng et al., 1997). As discussed below, this portion of the composite signal may be active at the mRNA level rather than at the protein or polypeptide level, because frameshift mutations that disrupt the sequence of the resulting translated protein can nevertheless be effective in the targeting process.

The second portion of the targeting signal is residues 15-100 of YopE. The sequence of residues 1-100 of YopE is given below:

M-K-I-S-S-F-I-S-T-S-L-P-L-P-A-S-V-S-G-S-S-S-V-G-E-M-S-G-R-S-V-S-Q-Q-K-S-D-Q-Y-A-N-N-L-A-G-R-T-E-S-P-Q-G-S-S-L-A-S-R-I-I-E-R-L-S-S-M-A-H-S-V-I-G-F-I-Q-R-M-F-S-E-G-S-H-K-P-V-V-T-P-A-L-T-P-A-Q-M-P-S-P-T (SEQ ID NO: 1).

Also included within the scope of the present invention are methods employing targeting signals that include amino acid substitutions or mutations within the second portion of the targeting signal. In particular, the conservative amino acid substitutions can be any of the following: (1) any of isoleucine, leucine, and valine for any other of these amino acids; (2) aspartic acid for glutamic acid and vice versa; (3) glutamine for asparagine and vice versa; and (4) serine for threonine and vice versa.

Other substitutions can also be considered conservative, depending upon the environment of the particular amino acid. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can be alanine and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the different pK's of these two amino acid residues or their different sizes are not significant. Still other changes can be considered "conservative" in particular environments. For example, if an amino acid on the surface of a protein is not involved in a hydrogen bond or salt bridge interaction with another molecule, such as another protein subunit or a ligand bound by the protein, negatively charged amino acids such as glutamic acid and aspartic acid can be substituted for by positively charged amino acids such as lysine or arginine and vice versa. Histidine (H), which is more weakly basic than arginine or lysine, and is partially charged at neutral pH, can sometimes be substituted for these more basic amino acids. Additionally, the amides glutamine (Q) and asparagine (N) can sometimes be substituted for their carboxylic acid homologues, glutamic acid and aspartic acid. Accordingly, processes employing targeting signals that contain conservative amino acid substitutions within residues 15-100 of the composite targeting signal are within the scope of the present invention.

Regarding the first portion of the composite targeting signal, Although Applicants do not intend to be bound by this theory, there is evidence that this first portion of the composite targeting signal is recognized at the mRNA level instead of, or possibly in addition to, the protein or polypeptide level (Anderson & Schneewind, 1997). The sequence of the mRNA encoding yopE, beginning at the point of initiation of translation, is AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCAUCAGUGUC AGGA (SEQ ID NO: 2).

Frameshift mutations that delete one nucleotide or add two nucleotides do not abolish the activity of the first portion of the composite targeting signal (Anderson & Schneewind, 1997). However, if the frameshift mutations result in a highly hydrophobic amino-terminal portion of the yopE protein, the activity of the first portion of the targeting

signal may be reduced or abolished. Therefore, also within the scope of the present invention are processes employing targeting signals whose first portion is encoded by a mRNA including therein SEQ ID NO: 2 or frameshift mutations of SEQ ID NO. 2 with the proviso that the hydrophobicity of the segment of the protein encoded by the mRNA including therein any frameshift mutation of SEQ ID NO: 2 is substantially equivalent to that of the hydrophobicity of the segment of the protein encoded by SEQ ID NO: 2. This mRNA has a structure of two stems with loops (Anderson & Schneewind, 1997).

Mutations that abolish translation may be located either within the first loop or its adjacent base pairs; the remainder of the sequence of the mRNA may be more resistant to the destruction or alteration of the function of the first portion of the signal by mutation. Therefore, also within the scope of the present invention are processes employing targeting signals whose first portion is encoded by a mRNA including the sequence within SEQ ID NO: 2 that is part of the first loop. This segment of mRNA has the sequence AAAAUAU. The first and last residues of this segment are base-paired.

Because of the pathogenicity of many Gram-negative bacteria, the translocation process described in the present application represents a novel site for antibiotic action. Accordingly, a process for blocking the introduction of bacterial proteins into eukaryotic cells is within the scope of the present invention. This process can involve inhibiting the transcription or translation of the mRNA for the YopE protein or an analogous protein with antisense technology, as described in J.A.H. Murray, ed., "Antisense RNA and DNA" (Wiley-Liss, New York, 1992), incorporated herein by this reference.

## EXAMPLE

The invention is illustrated by the following Example. This Example is for illustrative purposes only and are not to be construed as limiting the scope of the invention in any manner.

Fractionation of HeLa Cells Infected with *Y. enterocolitica*

We developed an experimental scheme to fractionate infected tissue cultures and separate the eukaryotic cytosol from adherent bacteria. Digitonin interacts specifically with cholesterol containing membranes (Esparis-Ogando et al., 1994). We reasoned that this detergent might cause the selective solubilization of the eukaryotic plasma membrane but not of bacteria which lack cholesterol. To test this, HeLa cells were infected with *Y. enterocolitica* and incubated until signs of Yop toxicity could be observed. The growth medium was removed and centrifuged to sediment non-adherent bacteria from the extracellular medium. HeLa cells and attached *Yersiniae* were scraped off the culture flasks, extracted with digitonin and centrifuged to separate the bacteria and other insoluble material from the supernatant, containing eukaryotic cytosol. As a control, a duplicate sample of infected HeLa culture was treated with sodium dodecyl sulfate (SDS) to solubilize all eukaryotic and bacterial membranes. Protein in all fractions was precipitated with chloroform / methanol and analyzed by immunoblotting.

Digitonin treatment released farnesyl protein-transferase (FPT) (Reiss et al., 1990) from the cytosol of HeLa cells into the extract supernatant (Fig. 1A). In contrast, bacterial chloramphenicol acetyl-transferase (CAT) (Alton and Vapnek, 1979) was not solubilized by digitonin and sedimented with the bacteria. Treatment with SDS, however, released both FPT and CAT into the supernatant. No FPT was found in the extracellular medium, indicating that the plasma membrane of HeLa cells remained intact during the infection with *Y. enterocolitica*. Thus, digitonin treatment selectively disrupted the eukaryotic plasma membrane but not bacterial cells and was employed to examine the location of Yop proteins. YopE, YopH, YopM and YopN were found in the supernatant of digitonin extracted HeLa cells but not in the extracellular medium. Three Yops were secreted into the extracellular medium: YopB, YopD, and YopR. YopB and YopR were also observed in the pellet fraction of digitonin extracts, whereas YopD was present in all fractions examined. YopQ sedimented with the adherent bacteria into the pellet fraction of digitonin extracts.

These results suggested that YopE, YopH, YopM and YopN were located in the eukaryotic cytosol, whereas other Yops were either secreted (YopB and YopR) or remained associated with the bacteria (YopQ). Yop targeting might be accomplished only by

*Yersinia* that have attached to HeLa cells but not by non-adherent bacteria (Rosqvist et al., 1994; Petterson et al., 1996). To measure *Yersinia* adherence to HeLa cells, we counted bacteria in the extracellular medium and those present in detergent extracts by dilution and colony formation. Most bacteria (95%) were attached to HeLa cells during tissue culture infection. CAT, a constitutively expressed protein, was present in adherent as well as non-adherent bacteria (93% in the digitonin pellet and 7% in the media pellet). In contrast, SycE, the secretion chaperone of YopE in the cytoplasm of *Yersinia*, was found only in adherent bacteria that sedimented after digitonin extraction. Similar results were observed for all other Yops with the notable exception of YopB and YopD (Fig. 1A). These data suggested that nonadherent bacteria synthesized YopB and YopD, whereas the attachment of *Yersinia* to HeLa cells provided a signal for the expression of other Yops.

The Targeting Signal of YopE

Previous work suggested that the N-terminal 50 and 73 residues of YopE and YopH are sufficient to promote the targeting of adenylate cyclase fusions (Sory et al., 1995). However, about half of all adenylate cyclase fusion protein was located in the extracellular medium (Boland et al., 1996). Because this report was in conflict with our measurements of YopE and YopH localization, we examined the targeting of neomycin phosphotransferase (NPT) (Reiss et al., 1984) fusions to YopE with our fractionation assay (Fig. 2A). Hybrid proteins containing either full length YopE or residues 1-100 of YopE fused to NPT were found in the supernatant of digitonin extracted HeLa cells (34 and 8 1 % respectively), indicating that they had been targeted in a manner similar to wild-type YopE (Fig. 2B). In contrast, fusions containing YopE residues 1-50, 1-15 or NPT alone remained in the pellet of digitonin extracts (0% targeting for all three hybrids). None of the hybrid YopE-NPT proteins were secreted into the extracellular medium.

SycE Is Required for YopE Targeting

To discern whether SycE binding to YopE polypeptide was required for targeting, we employed *Y. enterocolitica* LC2 (*sycE1*) which lacks the cytoplasmic SycE chaperone (Cheng et al., 1997). This strain secretes YopE into the medium via its secretion

signal (codons 1-15) under low calcium conditions. However, when tested during HeLa cell infections, YopE, YopE-NPT and $YopE_{1-100}$-NPT remained in the pellet of digitonin extracts, indicating that neither targeting nor secretion had occurred (0% for each of the three strains) (Fig. 2C). The defect of the *sycE1* strain was specific for the targeting of YopE-NPT fusions, since YopM and YopH were still located in the HeLa cytosol. The targeting defect of strain LC2 was due to the mutation carried by the *SycE1* allele, because it could be complemented by a plasmid encoded wild-type allele.

The presence of YopE-NPT fusions in the cytosol of HeLa cells was visualized with anti-NPT staining and immunofluorescence confocal laser microscopy (Fig. 3). To define the eukaryotic cytosol, the HeLa cell plasma membrane was stained with Texas red-labeled wheat germ agglutinin. Targeting of YopE-NPT and $YopE_{1-100}$-NPT could be detected as Oregon green-staining of the HeLa cytosol. $YopE_{1-50}$-NPT, $YopE_{1-15}$-NPT or NPT alone yielded the same amount of background staining as a control culture infected with *Yersiniae* that did not express NPT. During infection with the *sycE1* mutant *Y. enterocolitica* LC2 strain, no fluorescent staining was observed for either YopE-NPT or $YopE_{1-100}$-NPT. Together these results demonstrate that SycE binding to YopE substrate is absolutely required for targeting of the polypeptide into the HeLa cell cytosol.

The Secretion Signal (Codons 1-15) and YopE Targeting

To examine the role of the secretion signal encoded within the first fifteen codons of *yopE* in targeting, we analyzed YopE-NPT mutants in which this signal had either been mutated or replaced. Hybrid proteins were examined for their ability to serve as substrates in both the targeting reaction as well as in the low calcium-induced secretion of either wild-type or *sycE1* mutant *Yersiniae* (Fig. 4). Replacement of the first fifteen codons of YopE with those of two non-secreted gene products, *E. coli* chloramphenicol acetyl-transferase (*cat*) (Takeshita et al., 1987) and β-galactosidase (*lacZ*) (Kalnins et al., 1983), did not affect secretion in low calcium-induced wild-type *Yersiniae* (Fig. 4). As reported previously, the $YopE_{+1}$-NPT fusion protein is also secreted by wild-type *Yersiniae* (Cheng *et al.*, 1997). The SycE dependence of YopE-NPT secretion was tested in *Y. enterocolitica* LC2. Low-calcium induced secretion was absolutely dependent on the binding of SycE to

YopE residues 15-100, because the three hybrid proteins remained in the cytoplasm of the *sycE1* mutant strain (Fig. 4). When tested during the infection of HeLa cells, all three mutant YopE-NPT fusions sedimented with the bacteria after digitonin extraction (Fig. 4). Thus, the binding of SycE to YopE residues 15-100 is not sufficient for the targeting reaction and some property of the first fifteen codons or amino acids of YopE must also be recognized by the type III machinery.

The YopE Secretion Signal in YopN Mutant *Yersiniae*

Our result suggested that the secretion signal of YopE alone may not be functional during the infection of HeLa cells by *Y. enterocolitica*. In contrast to low calcium-induced secretion, in which all Yop proteins were secreted into the medium, the export of Yops during the infection of HeLa cells appeared to be regulated and directed to three distinct locations. *yopN* mutants of *Y. pseudotuberculosis* or *Y. enterocolitica* are known to secrete large amounts of Yops into the extra-cellular medium even during tissue culture infections (Forsberg et al., 1991; Petterson et al., 1996; Boland et al., 1996). To examine a possible role of YopN in preventing the secretion of YopE, we constructed *Y. enterocolitica* strain VTLI (*yopN1*). As expected, this mutant strain was temperature sensitive for growth and secreted Yops into the culture medium at 37°C even in the presence of calcium. During the infection of HeLa tissue cultures, *Y. enterocolitica* VTLI secreted all Yops into the extracellular medium (Fig. 1B). Some YopE, YopH and YopM could be found in the supernatant of digitonin extracts, suggesting that the *yopN1* strain may still be able to promote Yop targeting. A plasmid encoded wildtype *yopN* allele restored the fractionation pattern of Yops to that observed for *Y. enterocolitica* W22703, indicating that the phenotype of strain VTLI was due to the mutation carried by the *yopN1* allele.

To test whether the secretion signal of YopE was functional in the *yopN* mutant, the location of YopE-NPT fusions was examined during the infection of HeLa cells with *Y. enterocolitica* VTLI (Fig. 2D). YopE-NPT, $YopE_{1-100}$-NPT, $YopE_{1-50}$-NPT, and $YopE_{1-15}$-NPT were secreted into the extra-cellular medium. As a control, NPT alone as well as YopE-NPT (pDA72), harboring a defective secretion signal, sedimented with the *yopN1* mutant bacteria. Thus, during the infection of HeLa cells the secretion signal located

within the first 15 codons of YopE is functional in *yopN* mutants but not in wildtype *Yersiniae*.

Electron Microscopic Detection of YopE-NPT Fusions

Digitonin fractionation alone cannot distinguish between intra-bacterial Yops and those that might be located on the surface of either bacterial or eukaryotic cells. For example, YopE-NPT fusions that sedimented after digitonin extraction ($YopE_{1-50}$, $YopE_{1-15}$) could be located either in the cytoplasm or on the surface of *Yersiniae*. If the latter were true, such a result would favor the two step translocation model of Yops, whereas the cytoplasmic location of these NPT fusions would indicate that targeting occurred by a different mechanism. The location of YopE-NPT fusions was measured with electron microscopy. When infected with *Y. enterocolitica* expressing YopE-NPT, NPT-specific immuno-gold particles were detected in the cytosol of HeLa cells and in the bacterial cytoplasm, but not on the surface of *Yersiniae* (Fig. 5 and Table 1). $YopE_{1-15}$-NPT was found in the bacterial cytoplasm but not on cell surfaces or in the HeLa cytosol. Similarly, when infected with the *sycE1* mutant *Y. enterocolitica* LC2, immuno-gold staining of YopE-NPT was observed only in the bacterial cytoplasm but not in the cytosol of HeLa cells or on cell surfaces. Together these data suggested that YopE-NPT fusions that were not targeted into the HeLa cytosol remained within the bacterial cytoplasm.

**Table 1**. Immuno- electron microscopic detection of YopE NPT fusion proteins.

| | Average number of protein A gold particles[a] | | |
|---|---|---|---|
| *Y. enterocolitica* strains[b] | Bacterial surface | Bacterial cytoplasm | $\mu m^2$ of HeLa cytoplasm |
| W22703, pDA36 | 0.6 | 12.2 | 1.6 |
| W22703, pDA46 | 0.8 | 19.0 | 0.8 |
| LC2 (sycE]), pDA36 | 0.3 | 5.4 | 0.4 |
| W22703 (no plasmid) | 0.3 | 3.4 | 0.9 |

[a]YopE-NPT fusion proteins were detected with anti-NPT followed by protein A-gold conjugate staining. Gold particles were counted and averaged per bacterium or $cm^2$ of HeLa cytoplasm. Data were gathered from 25 bacteria.
[b]fusion proteins were expressed from low-copy number plasmids, either full length YopE-NPT (pDA36) or $YopE_{1-15}$-NPT (pDA46).

Discussion

Examination of the signals for the secretion of Yop proteins in low-calcium induced cultures lead to the identification of two distinct modes of type III secretion (Cheng et al., 1997). One pathway recognizes a signal encoded within the first fifteen codons of all Yops examined thus far (Sory et al., 1992; Schesser et al., 1996; Anderson and Schneewind, 1997). In most cases, this signal can be mutated by the insertion or deletion of nucleotides immediately following the start codon without loss of function (Anderson and Schneewind, 1997). This result, taken together with the fact that Yop proteins do not share either peptide sequence homology or common amino acids, suggests that a property of the mRNA may be recognized by the type III machinery. However, during HeLa cell infections the YopE secretion signal alone did not lead to the secretion of fusion proteins into the extracellular medium. This result does of course not exclude the possibility that similar signals of other Yops may lead to secretion during infection.

The second mode of type III secretion requires the binding of SycE to residues 15-100 of YopE (Cheng et al., 1997). This interaction is also sufficient for the secretion of reporter fusions in low-calcium induced cells. Here we report that SycE binding to YopE is absolutely necessary for YopE targeting into the HeLa cytosol. Forsberg and co-workers have investigated the SycE/YerA requirement with immunofluorescent microscopy and reported a low level targeting of YopE in a *yerA* mutant of *Y. pseudotuberculosis* (Frithz-Lindsten et al., 1995). Our experiments compared the digitonin fractionation with immunofluorescent microscopy and we find that the latter is less sensitive. Hence, even with the more sensitive digitonin fractionation technique we cannot detect YopE in the HeLa cytosol during infection with *sycE1* mutant *Yersiniae* and conclude that the binding of SycE is an absolute requirement for the targeting of YopE. SycE binding is not sufficient for YopE targeting which also requires the first fifteen codons or amino acids in addition to the SycE binding site (YopE residues 15-100). These results indicate that the substrate

requirement for YopE targeting differs significantly from that identified for type III secretion by low calcium-induced *Yersiniae*.

Our data are in disagreement with those of Cornelis and co-workers who reported that during the infection of macrophages with *Y. enterocolitica*, Yop-Cya fusions are found in the tissue culture medium as well as in the eukaryotic cytosol (Boland et al., 1996). The YopE secretion signal (codons 1-15) was presumed to direct fusion proteins into the extracellular medium as an intermediary step of Yop targeting. This assumption led to the proposal of a two step translocation mechanism for Yop targeting and to the conclusion of a modular organization of secretion and translocation signals within Yop proteins (Sory et al., 1995). We employed digitonin fractionation of *Yersinia* infected HeLa cells and report that YopE, H, M, and YopN are localized to the eukaryotic cytoplasm but not to the extracellular medium. Another surprising finding of this experimental technique is the secretion of YopB, YopD and YopR into the extra-cellular medium. YopB and YopD are absolutely required for the targeting of other Yops but it is not clear how this could be accomplished by their secretion into the medium. One caveat of our fractionation technique is that Yop proteins might be targeted into HeLa cells and later assume a digitonin insoluble state, such as membrane integration or import into the eukaryotic nucleus. For example, the nuclear transcription factor TFIID (Sawadogo and Roeder, 1985) was not solubilized by digitonin extraction (Fig. 1). To uncover the precise location of those Yops that sediment with the bacteria during digitonin fractionation, will require additional experimentation such as electron microscopy. YopQ (YopK) has been located by immunofluorescence microscopy to *Y. pseudotuberculosis* during HeLa cell infections (Holmstrom et al., 1997), however its subcellular location is still unknown. Adenylate cyclase fusions to YopN did not yield an increase of cAMP during the infection of macrophages by *Y. enterocolitica* (Boland et al., 1996). In contrast, we report that YopN was solubilized in part by digitonin extraction. YopN-NPT fusions did not acquire such digitonin solubility, suggesting that the discrepancies between the two experimental approaches can be explained by the aberrant subcellular location of Yop fusion proteins.

We propose a new model in which YopE is targeted directly from the bacterial cytoplasm into the cytosol of HeLa cells. Translocation across three membranes may be achieved by a type III secretion channel spanning the bacterial inner and outer membranes, which may be extended by other, hitherto unknown, proteins into the eukaryotic cytosol. Because the YopB and YopD proteins are absolutely required for targeting (Hakansson et al., 1996a), it is tempting to speculate that these polypeptides might fulfill such a role. The substrate requirements for YopE targeting are residues 1-100 bound to SycE chaperone. Because none of the targeted Yops (YopE, YopH, YopM, YopN and YopO) display sequence homology we think it is likely that the Syc proteins play an important role in substrate recognition. For example, a conserved C-terminal sequence element (Wattiau *et al.*, 1996) or other features of these secretion chaperones could be recognized by the type III machinery. If chaperone delivery to the type III machinery were a universal feature of Yop targeting, one would predict the existence of other Syc proteins (for YopM, YopN and YopO) that have not yet been identified (Wattiau *et al.*, 1994).

All Yop proteins require the type III machinery for their export from the bacterial cytoplasm (Allaoui et al., 1995). One explanation for the different locations of Yop proteins, i.e. the medium, HeLa cytosol or associated with the bacteria, would be that their structural genes are expressed at different times during tissue culture infection. Hence, Yop proteins that are synthesized once the Yersinia have docked on the surface of HeLa cells might be directed into the eukaryotic cytosol, whereas others, that were expressed prior to attachment, might be secreted. An alternative explanation for the different locations of Yop proteins would be that Yersinia switch the mode of substrate recognition for the type III machinery. For example, Yops might first be secreted into the medium by an mRNA encoded signal. Later during infection, perhaps after docking of the bacteria on the surface of HeLa cells, type III export may occur only if Yops are properly delivered by their chaperones. It is equally plausible that Yersinia employ both regulatory elements, gene expression and alternate modes of substrate recognition, to position their Yops proteins at different locations relative to the eukaryotic target cell.

Experimental Procedures

HeLa Cell Infections

*Yersinia* strains were grown in Luria broth at 26°C with 150 rpm shaking, diluted 1:20 into fresh media and incubated for another 2 hours ($OD_{600}$ 0.4). HeLa cells were grown in Dulbecco's minimal eagle medium (DMEM) supplemented with 10% FBS to confluency (2.5 x $10^7$ cells per 75 $cm^2$ flask). Cells were washed twice with 5 ml PBS, incubated in 10 ml DMEM for 30 minutes, infected with *Y. enterocolitica* [2.5 x $10^8$ bacteria, multiplicity of infection (MOI) 10] and incubated for 3 hours at 37°C, 5% $CO_2$. Media were collected by decanting and placed on ice. HeLa cells attached to the flasks were lysed by the addition of 10 ml PBS containing either 1% purified digitonin or 1% SDS and 10 mM EDTA. Detergent solutions were incubated for 20 min at room temperature with vigorous intermittent vortexing. Samples were sedimented by centrifugation at 20,000 g for 15 minutes. A 6.6 ml portion of supernatant was transferred to a new tube and the remainder discarded. The sediment was suspended in 10 ml of 1% SDS in PBS and a 6.6 ml portion transferred to a new tube. Protein was precipitated with methanol / chloroform (Wessel and Flugge, 1984), and suspended in 400 III sample buffer (10% glycerol, 1% SDS, 0.1% bromophenol blue, 5.5 M urea, 2% β-mercaptoethanol, 36 mM Tris-HCl, pH 6.8). Proteins were separated on SDS-PAGE, electrotransferred onto PVDF membrane, immunoblotted with specific antiserum, and identified as a chemiluminescent signal on X-ray film.

Immunofluorescence

HeLa cells (2 x $10^5$) were grown in DMEM on cover slips in a 24 well plate for 48 hours at 37°C, 5% $CO_2$. Cells were washed twice with PBS, covered with 1 ml DMEM, and infected with 8 x $10^6$ bacteria (MOI of 10) for 3 hours. Samples were first washed with PBS and then fixed with 3.7% formaldehyde in PBS for 20 minutes. The reaction was quenched by washing in PBS and then adding 0.1 M glycine for 5 minutes. HeLa cells were permeabilized with 1% Triton in PBS for 30

minutes. Samples were blocked for nonspecific staining with 5% nonfat milk, 0.05% Tween 20 in PBS for 15 minutes followed by incubation with anti-NTT (1:100 dilution) for 20 minutes. Samples were washed four times with PBS 0.05% Tween 20 for 5 minutes each and incubated with goat anti-rabbit IgG Oregon 488 green-conjugate as well as wheat germ agglutinin Texas Red-conjugate (Molecular Probes, both diluted 1:500) for 20 minutes. Samples were washed four times, dried for 1 hour and viewed under a Leica confocal laser microscope.

Immuno-Electron Microscopy

HeLa cells were infected with *Yersinia* and incubated for 3 hours at 37°C, 5% $CO_2$ as described above. Cells were washed with 10 ml of PBS, scraped off the plate and fixed overnight at 4°C in 2% formaldehyde, 6% sucrose in PBS. Samples were washed twice with PBS and the remaining formaldehyde was quenched with 0.01 M glycine in PBS for 10 minutes. The samples were dehydrated through a graded series of ethanol, placed in resin through a graded series of ethanol-LR White mixtures and baked overnight at 55°C to dry the resin. The embedded cells were cut with an ultra-microtome and thin sections were collected on formvar-coated nickel grids. Samples were immuno-stained at room temperature by floating the grids on a series of 50 µl droplets of different solutions: blocking in 50 mM HEPES, 0.3 M NaCl, 0.05% $NaN_3$, 1 % BSA, 0.01 % cold water fish skin gelatin for 30 min, anti-NPT 1:5 in blocking solution for 1 hour, 7 washes with 50 mM HEPES, 0.3 M NaCl, 0.05% $NaN_3$, protein A-colloidal gold conjugate (9 nm particles) 1:50 in blocking solution for 1 hour, and finally another 7 washes. Samples were fixed in 2% glutaraldehyde for 10 min, stained first with 2% uranyl acetate for 10 minutes and then with Reynold's lead for 1 minute. Grids were washed in water and viewed under a transmission electron microscope.

Antisera

Yops were precipitated from the supernatant of low-calcium induced *Y. enterocolitica* strain 8081 cultures (Portnoy *et al.*, 1981) with ammonium sulfate

(46%). Precipitated Yops were suspended in 6 M guanidine-HCl, 0.01 M phosphate buffer, 10 mM DTT and separated by reverse phase HPLC on C8 column (Anderson and Schneewind, 1997). YopE, H, M and YopN were purified in this manner. The coding sequences for YopB, D and YopR were PCR amplified with primers specifying abutted BamHl restriction sites for cloning into the pQE vectors (Qiagen). Histidine tagged polypeptides were overexpressed in E. coli and purified by affinity chromatography on NI-NTA followed by separation on reverse phase HPLC. Purified polypeptides were injected into rabbits for antibody production, whereas antisera against FPT (Signal Transductions), NPT (5'->3') and TFIID (Oncogen Research) were purchased.

Plasmids and Strains

The pDA plasmids have been described elsewhere (Anderson and Schneewind, 1997). Plasmids pVL33 and pVL35 were generated by inserting annealed oligonucleotides specifying the codons 1-15 of either *cat* or *lacZ* between the *Nde*I and *Kpn*I sites of pDA 139. Plasmids were sequenced for confirmation and transformed into *Yersinia* strains. The *Y. enterocolitica* strains W22703 (Cornelis and Colson, 1975) and LC2 (*sycE1*) (Cheng et al., 1997) have been described previously. The *yopN1* mutant strain has a stop codon followed by a nucleotide insertion and BamHI site inserted at codon four of the *yopN* gene (Forsberg et al., 1991). The *yopN1* mutation was introduced by allele replacement following a standard protocol (Cheng *et al.*, 1997). Procedures to measure Yop secretion by *Yersinia* strains have been previously reported (Anderson and Schneewind, 1997).

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## ADVANTAGES OF THE PRESENT INVENTION

The present invention provides a widely applicable and efficient method for translocating cloned proteins into eukaryotic cells. The method does not depend on particular structural features of the protein to be translocated and thus can be used with virtually any protein to be translocated, as long as a suitable fusion protein can be prepared. The method can also be used with a broad range of eukaryotic cells. The method is rapid and efficient. The method of the present invention also preserves the three-dimensional structure and activity of the translocated proteins and is performed under conditions that do not expose such proteins to denaturing or destabilizing conditions. The method is also suited to a broad range of applications, including screening of the proteins that have been translocated.

Although the present invention has been described herein in detail with respect to certain preferred embodiments thereof, it will be clear to those skilled in the art that various changes and modifications can be made without departing from the invention. Therefore, the scope of the invention is to be determined by the following claims.

SEQUENCE LISTING

<110> Vincent T. Lee
Olaf Schneewind

<120> A METHOD FOR THE TARGETING OF PROTEINS
PRODUCED BY YERSINIA INTO THE CYTOSOL OF EUKARYOTIC CELLS

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What is claimed is:

1. A method for delivering a peptide to the cytosol of a eukaryotic cell comprising the steps of:

(a) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (1) a composite YopE targeting signal; and (2) a polypeptide to be translocated, the nucleic acid segment being expressed in *Yersinia* cells;

(b) expressing the nucleic acid segment in *Yersinia* cells;

(c) contacting the *Yersinia* cells expressing the nucleic acid segment with eukaryotic cells in the presence of functional SycE chaperone activity so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells.

2. The method of claim 1 where the *Yersinia* cells are selected from the group consisting of *Yersinia enterocolitica* and *Y. pseudotuberculosis*.

3. The method of claim 2 wherein the *Yersinia* cells are *Y. enterocolitica*.

4. The method of claim 1 wherein the eukaryotic cells are animal cells.

5. The method of claim 4 wherein the animal cells are mammalian cells.

6. The method of claim 5 wherein the mammalian cells are human cells.

7. The method of claim 4 wherein the eukaryotic cells are of a cell type selected from the group consisting of fibroblasts, epithelial cells, and leukocytes.

8. The method of claim 1 wherein the eukaryotic cells are plant cells.

9. The method of claim 1 wherein the polypeptide is selected from the group consisting of an antibody, an enzyme, a receptor protein, a hormone, and a toxin.

10. The method of claim 1 wherein the polypeptide further comprises a reporter segment.

11. The method of claim 1 wherein the composite targeting signal comprises the amino acid sequence M-K-I-S-S-F-I-S-T-S-L-P-L-P-A-S-V-S-G-S-S-S-V-G-E-M-S-G-R-S-V-S-Q-Q-K-S-D-Q-Y-A-N-N-L-A-G-R-T-E-S-P-Q-G-S-S-L-A-S-R-I-I-E-R-L-S-S-M-A-H-S-V-I-G-F-I-Q-R-M-F-S-E-G-S-H-K-P-V-V-T-P-A-L-T-P-A-Q-M-P-S-P-T (SEQ ID NO: 1).

12. The method of claim 1 wherein the first fifteen amino acids of the composite targeting signal are encoded by a mRNA sequence of AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCA (SEQ ID NO: 2).

13. The method of claim 1 wherein the first fifteen amino acids of the composite targeting signal are encoded by a mRNA sequence that is selected from the group consisting of:

(a) the mRNA sequence of AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCA (SEQ ID NO: 2); and

(b) a mRNA sequence that is modified from the sequence AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCA by a frameshift mutation, with the proviso that the hydrophobicity of the segment of the protein encoded by the mRNA including therein any frameshift mutation occurring within the sequence AUGAAAAUAUCAUCAUUUAUUUCUACAUCACUGCCCCUGCCGGCA is substantially equivalent to the hydrophobicity of the segment of the protein encoded by SEQ ID NO: 2.

14. The method of claim 1 wherein the first fifteen amino acids of the targeting signal are encoded by a mRNA including the sequence AAAAUAU that is part of the first loop of SEQ ID NO: 2.

15. The method of claim 1 wherein the first fifteen amino acids of the composite targeting signal are M-K-I-S-S-F-I-S-T-S-L-P-L-P-A (SEQ ID NO: 1).

16. The method of claim 1 wherein residues 16-100 of the composite targeting signal are selected from the group consisting of:

(a) S-V-S-G-S-S-S-V-G-E-M-S-G-R-S-V-S-Q-Q-K-S-D-Q-Y-A-N-N-L-A-G-R-T-E-S-P-Q-G-S-S-L-A-S-R-I-I-E-R-L-S-S-M-A-H-S-V-I-G-F-I-Q-R-M-F-S-E-G-S-H-K-P-V-V-T-P-A-L-T-P-A-Q-M-P-S-P-T, which are residues 16-100 of SEQ ID NO: 1; and

(b) sequences that include are modified from residues 16-100 of SEQ ID NO: 1 by one or more of the following conservative amino acid substitutions:

(i) isoleucine for either valine or leucine;

(ii) valine for either isoleucine or leucine;

(iii) leucine for either valine or isoleucine;

(iv) aspartic acid for glutamic acid;

(v) glutamic acid for aspartic acid:

(vi) glutamine for asparagine;

(vii) asparagine for glutamine;

(viii) serine for threonine; and

(ix) threonine for serine.

17. A method for blocking the introduction of a bacterial protein translocated by a translocation system in a Gram-negative bacterium employing a targeting signal into a eukaryotic cell comprising inhibiting the transcription or translation of mRNA for a protein of the translocation system having protein targeting activity by administering at least one antisense oligonucleotide to inhibit the transcription or translation of the protein having protein targeting activity.

18. The method of claim 17 wherein the Gram-negative bacterium is a *Yersinia* species and the protein of the translocation system is the *Yersinia* YopE protein.

19. A method for screening for the activity of a translocated polypeptide in a eukaryotic cell comprising the steps of:

(a) providing a translocated polypeptide in a eukaryotic cell by:

(i) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (1) a composite YopE targeting signal; and (2) a polypeptide to be translocated, the nucleic acid segment being expressed in *Yersinia* cells;

(ii) expressing the nucleic acid segment in *Yersinia* cells;

(iii) contacting the *Yersinia* cells expressing the nucleic acid segment with eukaryotic cells in the presence of functional SycE chaperone activity so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells; and

(b) screening for the activity of the translocated polypeptide by detecting the result of an activity of the polypeptide in the eukaryotic cell.

20. The method of claim 19 wherein the detection of the result of the activity of the polypeptide in the eukaryotic cell is performed by a method selected from the group consisting of detection of an enzymatic activity, detection of an antibody activity, and detection of a specific binding activity.

21. A method for delivering a peptide to the cytosol of a eukaryotic cell comprising the steps of:

(a) providing a nucleic acid segment encoding a fusion protein, the fusion protein including: (1) a targeting signal effective for targeting in a Gram-negative bacterium possessing a Type III translocation mechanism homologous or substantially homologous to the translocation mechanism of *Yersinia*; and (2) a

polypeptide to be translocated, the nucleic acid segment being expressed in the cells of the Gram-negative bacterium;

(b) expressing the nucleic acid segment in the Gram-negative bacterial cells;

(c) contacting the Gram-negative bacterial cells expressing the nucleic acid segment with eukaryotic cells in the presence of functional chaperone activity homologous or substantially homologous to SycE chaperone activity of *Yersinia* so that the fusion protein including the polypeptide is translocated to the cytosol of the eukaryotic cells.

22. The method of claim 21 wherein the Gram-negative bacterium is selected from the group comprising *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Pseudomonas spp.*, and *Xanthomonas spp.*

23. The method of claim 21 wherein the targeting signal includes a second portion that is specific for a protein that has substantial homology with the SycE protein of *Yersinia* at its carboxy-terminal end and has chaperone function.

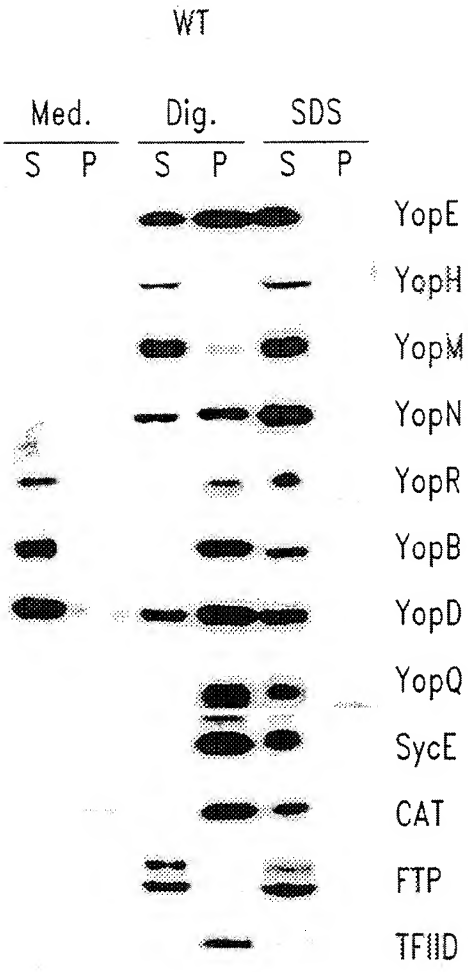
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*Fig. 1A*

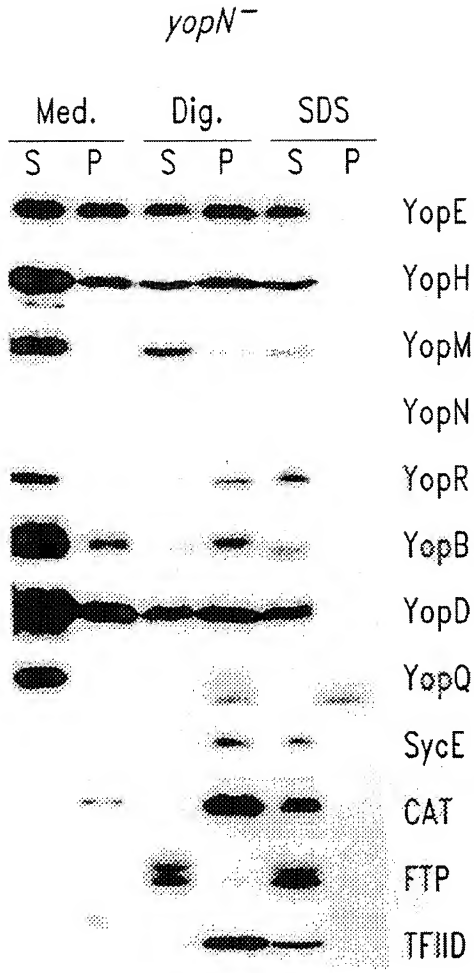
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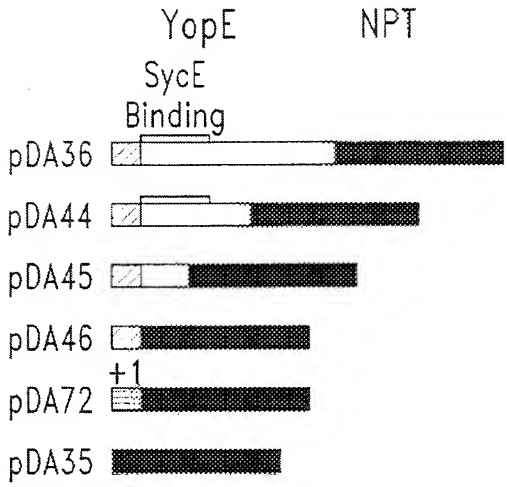
*Fig. 1B*

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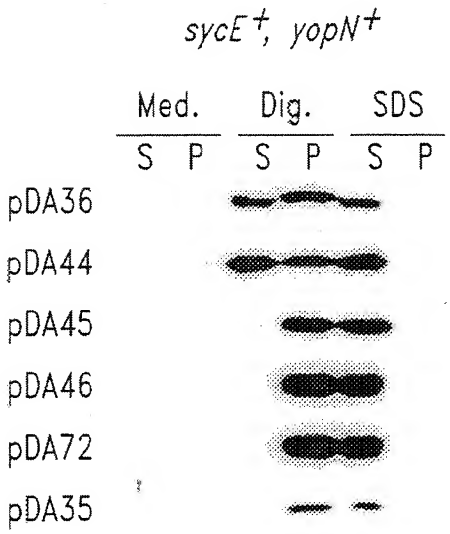


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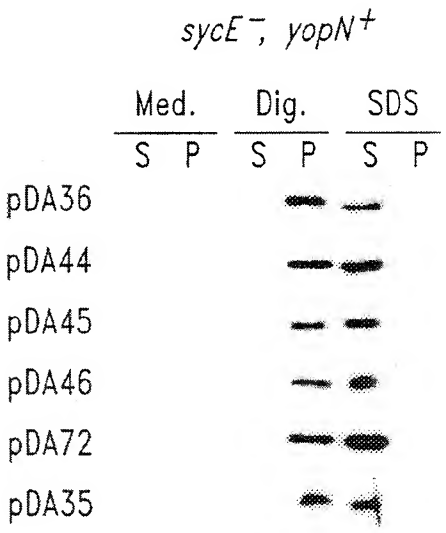


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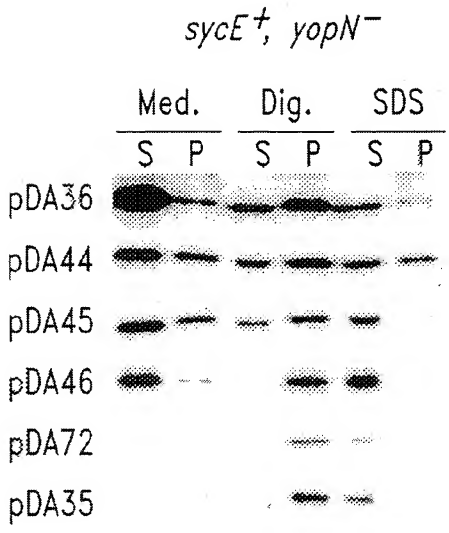


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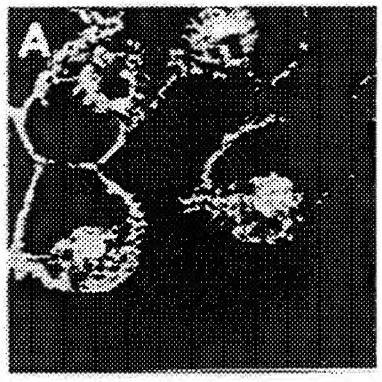


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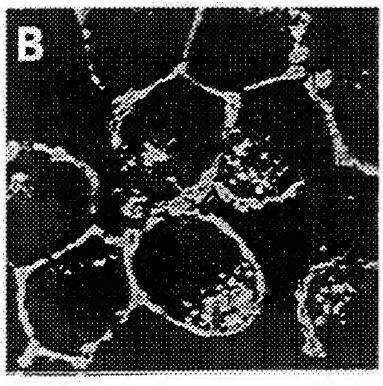


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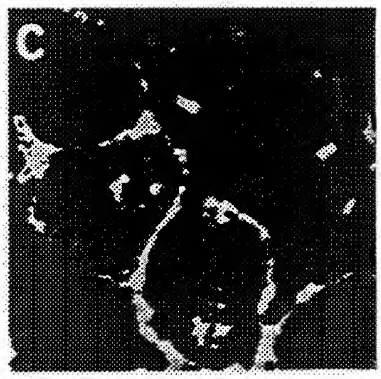


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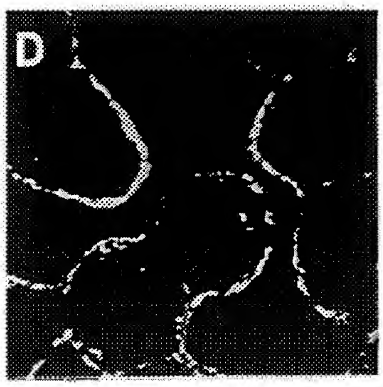


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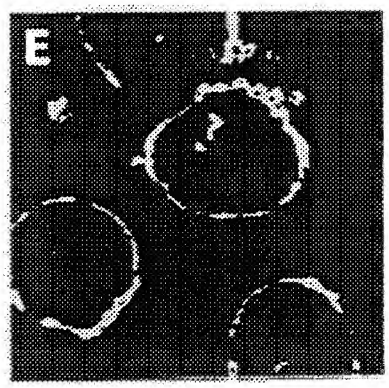


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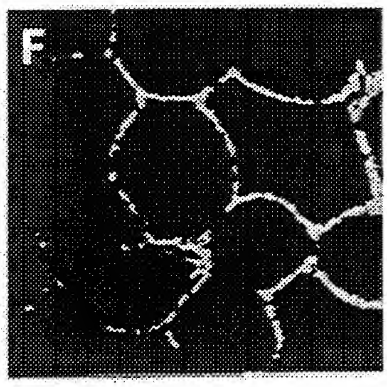


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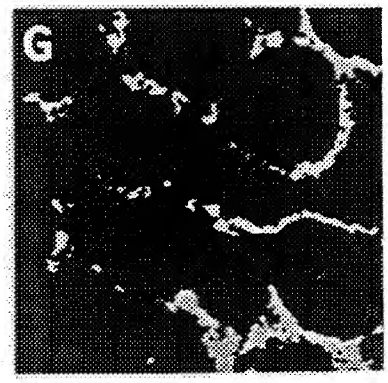


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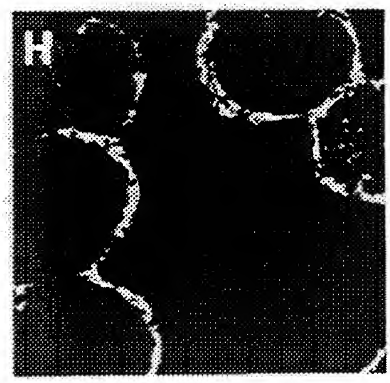


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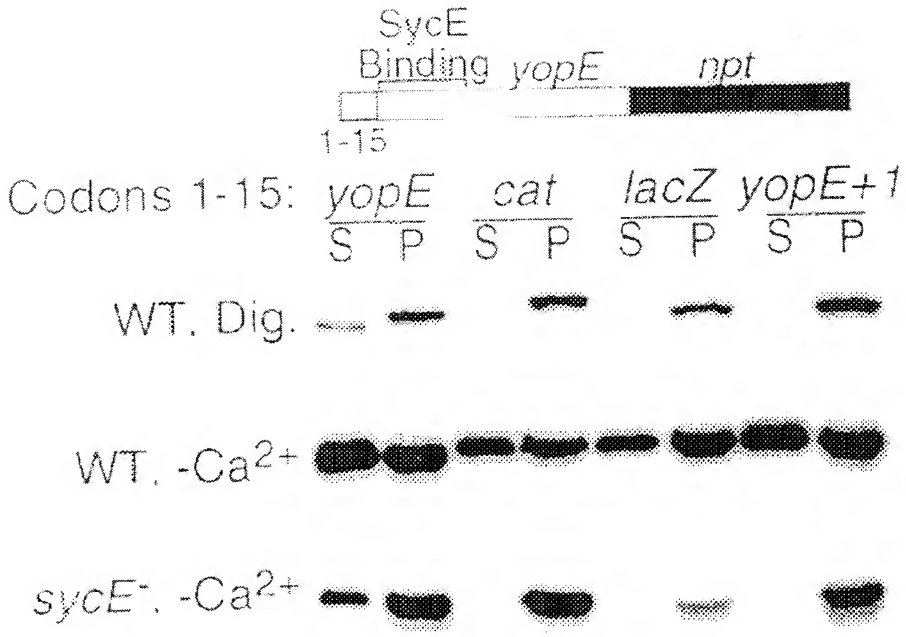
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sycE-, -Ca2+

*Fig. 4*

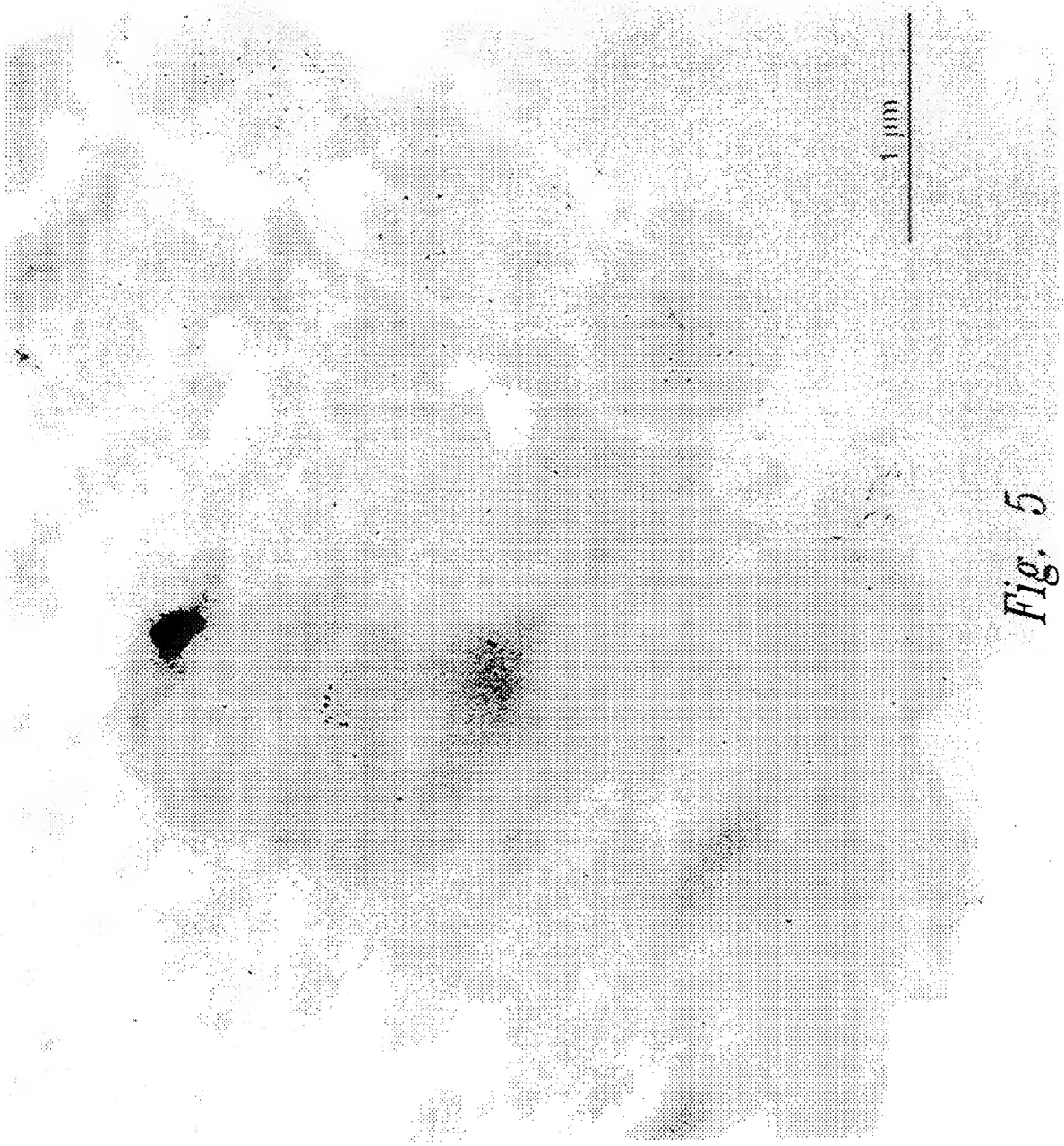


Fig. 5

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08209

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 48/00; A01N 63/00; C12N 15/00; C12Q 11/68
US CL :514/44; 424/93.21; 435/69.1, 325, 455, 6
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/93.21; 435/69.1, 325, 455, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | FRITHZ-LINDSTEN et al. The chaperone-like protein YerA of Yersinia pseudotuberculosis stabilizes YopE in the cytoplasm but is dispensible for targeting to the secretion loci. Molecular Microbiology. 1995, Vol. 16, No. 4, pages 635-647, especially page 635, column 1. | 1-10, and 17-23 |
| Y | SORY et al. Translocation of a hybrid YopE-adenylate cyclase from Yersinia enterocolitica into HeLa cells. Molecular Microbiology. 1994, Vol. 14, No. 3, pages 583-594, especially page 589. | 1-10, and 17-23 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>30 JUNE 1999 | Date of mailing of the international search report<br>19 AUG 1999 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>DAVE NGUYEN<br>JOYCE BRIDGERS<br>PARALEGAL SPECIALIST<br>CHEMICAL MATRIX<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08209

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | SORY et al. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. Proc. Natl. Acad. Sci. USA. December 1995, Vol. 92, pages 11998-12002, especially page 11998. | 1-10, and 17-23 |
| Y | ANDERSON et al. A mRNA Signal for the Type III Secretion of Yop Proteins by Yersinia enterocolitica. Science. 07 November 1997, Vol. 278, pages 1140-1143, especially page 1142. | 1-10, and 17-23 |
| Y | HOLMSTROM et al. YopK of Yersinia Pseudotuberculosis controls translocation of Yop effectors across the eukaryotic cell membrane. Molecular Microbiology. 1997, Vol. 24, No. 1, pages 73-91, especially page 73. | 1-10, and 17-23 |
| Y | SCHESSER et al. Delineation and Mutational Analysis of the Yersinia pseudotuberculosis YopE Domains which mediate translocation across Bacterial and Eukaryotic Cellular Membranes. Journal of Bacteriology. December 1996, Vol. 178, No. 24, pages 7227-7233, especially page 7227. | 1-10, and 17-23 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08209

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 11-16
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 11-16 recite specific sequences, e.g., SEQ ID NOS: 1, 2, and the sequence recited in item (b) of claim 13, however, neither the disclosure nor the priority application contain the electronic version of the recited sequences. Thus, no meaningful search could be carried out.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.